Supplemental Information

Supplemental Figures

Supp. Figure 1. Western blot of total PARP1.

Supp. Figure 2. PARP1 and other proteins associated with AZD2281 response. IC50s for AZD2281 at 5 days (see Fig 5B) were correlated with the baseline protein expression in lung cancer cell lines by Spearman correlation. Proteins significantly correlated at a false discovery rate of <5% (corresponding p-value <0.016) are shown.

Supp. Figure 3. Effect of PARP inhibition in combination with standard chemotherapy. H82 and H69 cells were treated with cisplatin and etoposide alone or in combination with AZD2281.

Materials and Methods

DNA fingerprinting to confirm cell line identity

DNA from 5-6 x 10^6 cells was isolated using a QIAamp DNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. DNA was eluted in 100 µl of elution buffer (Buffer AE, Qiagen). The concentration of the eluted DNA was measured by the absorbance at 260 nm, and the purity of the eluted DNA was determined by the ratio of the absorbance at 260 nm to the absorbance at 280 nm. About 50 ng of DNA was used for DNA fingerprint analysis of short tandem repeat profiling (PowerPlex 1.2, Promega, Madison, WI) to authenticate each cell line. The analysis system used covers at least eight short tandem repeat loci. Fingerprinting results for each cell line were compared to reference fingerprints provided by Dr. Minna or the ATCC.

Preparation of Protein Lysates and Reverse-Phase Protein Array

Protein lysate was collected from subconfluent cultures after 24-hr in full-serum media (10% fetal bovine serum [FBS]), serum-starved media (0% FBS), or serum-stimulated media (24 hr of 0% FBS followed by 30 min of 10% FBS immediately before lysis).For total protein lysate preparation, media were removed, and cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing Complete protease and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany) and 1 mM Na_3VO_4. Lysis buffer (1% Triton X-100, 50 mM HEPES [pH 7.4], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM NaPPi, 10% glycerol, 1 mM PMSF, 1 mM Na_3VO_4, and 10 µg/mL aprotinin). Samples were vortexed frequently on ice and then centrifuged. Cleared supernatants were collected and protein quantified using a BCA kit (Pierce Biotechnology, Inc., Rockford, IL). (1)

RPPAs were printed from lysates as previously described (2). For each cell line, a serial dilution of 5 concentrations was printed with 10% of the samples replicated for quality control. Immunostaining was performed with an automated autostainer (BioGenex, San Ramon, CA). Each array was incubated with primary antibody (Supp. Table 7), and signal was detected using a catalyzed signal amplification system (DakoCytomation California, Inc., Carpinteria, CA). Primary antibodies were extensively validated via Western blots, where band quality and correlation of protein levels in previous RPPA experiments were determined, as previously described (1, 3)

RPPA Data Processing and Statistical Analysis

MicroVigene software (VigeneTech, Carlisle, MA) and an R package developed in house (4) were used to measure spot intensity. Protein levels were quantified by a

SuperCurve method as previously described (3). Data were logarithm-transformed (base 2). Median-control normalization was then applied to the dataset.

Distinct protein expression patterns present across the cell lines were assessed by unsupervised two-way hierarchical clustering using Pearson correlation distance between proteins (rows), Euclidean distance between cell lines (columns), and the Ward's linkage rule.and by first principal component analysis. An analysis of variance (ANOVA) model was applied on a protein-by-protein basis to identify differentially expressed proteins. F-statistics were used to calculate p values. The resulting p values were modeled using the beta-uniform mixture (BUM) model and used to determine a false discovery rate (FDR) cutoff to identify significantly differentially expressed proteins(5)(6). This approach models the resulting p values (computed from test statistics) and chooses an appropriate cutoff for the single test p-values by controlling the FDR, which is defined as the percentage of proteins called significant that are expected to turn out false. In the analysis, we used a FDR of 1% (corresponding p value=0.0388) plus and additional requirement that the fold difference between SCLC and NSCLC be >=1.5 (to try to minimize inclusion of proteins where difference in expression might be statistically significant, but were less likely to be biologically or clinically significant). Statistical analyses were performed using R statistical software (version 2.10.0).

Immunohistochemical Analysis for Total PARP1

The tissue microarray contained 96 neuroendocrine tumor specimens (13 SCLC, 19 LCNEC, 9 atypical carcinoids and 55 typical carcinoids), 49 NSCLCs (24 adenocarcinomas and 14 SCCs) collected between 1989 and 2005 after surgical

resection from patients under an IRB protocol and archived as formalin-fixed paraffin-embedded specimens in the lung cancer tissue bank at UTMDACC. TMAs were prepared using 1-mm-diameter cores in triplicate.

TMA histology sections (5 μm thick) were deparaffinized, hydrated, and antigen retrieval was performed using a decloaker with Dako Target Retrieval pH 6.0. Sections were incubated with 3% H_2O_2, followed by Serum Free Protein Block (DAKO). Slides were incubated at room temperature for 45 min with total PARP1 antibody (Thermo Fisher, Freemont, CA) and then for 30 min with the secondary antibody Envision+ Dual Link (DAKO). Following three washes, slides were incubated with Dako chromogen substrate for 5 min and counterstained with hemotoxylin for 5 min (13).

Nuclear expression was quantified using a four-value intensity score (0, none; 1+, weak; 2+, moderate; and 3+, strong) and the percentage (0-100%) of the extent of reactivity. A final expression score was obtained by multiplying the intensity and reactivity extent values (range, 0-300) (14). Cells lines with low (HCC827) or high (H82 and H524) PARP1 were used as positive controls.

Poly ADP-ribose (**PAR) Assay**

To evaluate the effect of AZD2281 (Astra Zeneca, Macclesfield, UK) on PARP1 activity in SCLC, we treated cells with 0, 0.1, 1, or 10 μM AZD2281. After 24 hrs, cell extracts were prepared, and poly ADP-ribose (PAR) levels were evaluated by ELISA, according to manufacturer's instructions (Trevigen, Inc., Gaithersburg, MD).

In Vitro Testing of PARP inhibitors and siRNA

For the MTS assay, cells in 5% RPMI media were seeded in a 96-well plate (2000 cells/well), incubated for 24 hr, and treated with AZD2281 (0–100 μM) in serum-containing media. On day 5, MTS was added for 1 hr. The absorbance was read at 490 nm on a plate reader. For the 14-day assay, cells were seeded in a 6-well plate (3000 cells/well for adherent cells; 30,000 cells/well for non-adherent cells). After 24 hr, AZD2281 or AG014699 was added at increasing concentrations (0, 0.16, 0.63, 2.5 and 10 μM). After 14 days, the cells were counted. For chemotherapy combination, cells were seeded in a 6-well plate (30,000 cells/well) and treated with 1 μM AZD2281 for 7 days. Cisplatin (0.56 or 1.68 μM) and etoposide (2.08 or 6.24 μM) (to reflect ratios of drug used in clinical practice) were added. After 7 days, the cells were counted. siRNA sequences were obtained from Ambion, Life Technologies (Calsbad, CA). Three distinct siRNA sequences targeting PARP were utilized in these experiments. These siRNA sequences targeting PARP included Silencer Select s1097 (sense: GGUGAUCGGUAGCAACAAAtt; antisense: UUUGUUGCUACCGAUCACCgt) s1098 (sense: CCAUCGAUGUCAACUAUGATT; antisense: CCAUCGAUGUCAACUAUGATT) s1099 (sense: GCAGCUUCAUAACCGAAGAtt; antisense: UCUUCGGUUAUGAAGCUGCtt). For additional siRNA targets, cells were transfected with control siRNA or a pool of four siRNAs for each target and then plated for cell proliferation assay (Thermo Scientific, Dharmacon RNAi, Waltham, MA). Viability was measured at day 1 and 5. Protein was collected and analyzed by Western analysis to confirm knockdown of targeted proteins.

Immunofluorescence staining for RAD51:

Immunofluorescence analysis for A549 cells was performed as described previously(15)**.** A549 cells were allowed to adhere onto glass coverslips overnight. The cells were irradiated at 4Gy using A Mark I ^{137}Cs irradiator (JL Shepherd and Associates, San Fernando, CA, USA) and cells were fixed post radiation at different time points (0, 1, 6, 18 and 24 hrs). For suspension cells, the cells were fixed onto glass slides using cytospin post radiation and fixed using 1% paraformaldehyde for 10 min followed by ethanol (70%) fixation for 10 minutes at room temperature. The cells were then treated with 0.1% NP40 in PBS for 20 min, washed with PBS four times and then blocked with 5% bovine serum albumin in PBS for 30 min. The cells were then incubated with anti–RAD51 antibody (Novus Biologicals) in 5% bovine serum albumin in PBS overnight. Next day, cells were incubated with FITC-labeled secondary antibody at a dilution of 1:300 in 5% BSA in PBS for 30 min. Cells then were incubated in the dark with 4 4’,6-diamidino-2-phenylindole (DAPI, 1 mg/mL) in PBS for 5 min, and coverslips were mounted on a slide with an antifade solution (Molecular Probes). Slides were examined using a Leica fluorescence microscope, and images were captured by a CCD camera and imported into Advanced Spot Image analysis software package. For each treatment condition, the percentage of cells expressing RAD51 foci were determined in minimum of 4 fields.

Supplemental Information References

1. Tibes R, *et al.* (2006) Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. *Mol Cancer Ther* 5(10):2512-2521.
2. Byers LA, *et al.* (2009) Reciprocal regulation of c-Src and STAT3 in non-small cell lung cancer. *Clin Cancer Res* 15(22):6852-6861.
3. Nanjundan M, *et al.* (Proteomic profiling identifies pathways dysregulated in non-small cell lung cancer and an inverse association of AMPK and adhesion pathways with recurrence. *J Thorac Oncol* 5(12):1894-1904.
4. Hu J, *et al.* (2007) Non-parametric quantification of protein lysate arrays. *Bioinformatics* 23(15):1986-1994.
5. Benjamini Y & Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to

Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 57(1):289-300.

6. Pounds S & Morris SW (2003) Estimating the occurrence of false positives and false negatives in microarray studies by approximating and partitioning the empirical distribution of p-values. *Bioinformatics* 19(10):1236-1242.
7. Zhou BB, *et al.* (2006) Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. *Cancer Cell* 10(1):39-50.
8. http://www.ncbi.nlm.nih.gov/geo/ (
9. Edgar R, Domrachev M, & Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30(1):207-210.
10. Barrett T, *et al.* (NCBI GEO: archive for functional genomics data sets--10 years on. *Nucleic Acids Res* 39(Database issue):D1005-1010.
11. Parkinson H, *et al.* (ArrayExpress update--an archive of microarray and high-throughput sequencing-based functional genomics experiments. *Nucleic Acids Res* 39(Database issue):D1002-1004.
12. http://www.ebi.ac.uk/microarray-as/a (
13. Tang X, *et al.* (Abnormalities of the TITF-1 lineage-specific oncogene in NSCLC: implications in lung cancer pathogenesis and prognosis. *Clin Cancer Res* 17(8):2434-2443.
14. Behrens C, *et al.* (Expression of interleukin-1 receptor-associated kinase-1 in non-small cell lung carcinoma and preneoplastic lesions. *Clin Cancer Res* 16(1):34-44.
15. Bhardwaj V, *et al.* (2012) C-Met Inhibitor MK-8003 Radiosensitizes c-Met-Expressing Non-Small-Cell Lung Cancer Cells With Radiation-Induced c-Met-Expression. *J Thorac Oncol*.